Fine mapping and cloning of the LepR3 blackleg (*Leptosphaeria maculans*) resistance gene in 'Surpass 400'

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Abstract

'Surpass 400' was released as a blackleg resistant cultivar containing a single dominant blackleg resistance gene (LepR3) from *Brassica rapa* ssp. sylvestris. This study focused on the mapping and cloning of this resistance gene using a map-based cloning strategy. A consensus map was developed using SRAP (Sequence related amplified polymorphism) markers and a double haploid (DH) population developed from a cross of 'Westar' and 'Zhongyou 821'. 908 F_2 and 2992 F_3 individuals of 'Westar' × 'Surpass 400' were used to follow the segregation of disease resistance. One Mendelian gene controlled the disease resistance to blackleg as showed by trait segregation. Starting with the development of an anchoring marker on the ultra-density map, a fine map was constructed for this resistance gene that has over a hundred SRAP markers showing co-segregation with the resistance gene. After sequencing 7 SRAPs that are linked closely to the resistance gene, a region flanking the closest marker was targeted to develop SNPs from 'Westar' and 'Surpass 400' according to the Arabidopsis and *B. rapa* sequence. These SNPs were used to screen the recombinant population and helped to locate the candidate gene for LepR3. This gene is a leucine-rich repeat transmembrane protein kinase that may be involved in pathogen recognition. The complete sequences of this gene in 'Westar' and 'Surpass 400' showed that there is an insertion in 'Surpass 400' and a several different SNPs between 'Surpass 400' and 'Westar' alleles.

Key words: *Brassica napus*, blackleg, resistance gene, mapping, cloning,

Introduction

Blackleg in canola and other cruciferous crops, caused by *Leptosphaeria maculans*, is a serious fungal disease that can cause major yield losses throughout the world (Oil World, 2000). Blackleg characteristics, genetics on plant resistance and pathogen-host relationships have been analyzed intensively for several decades and a considerable volume of valuable data has been collected. However, the avirulence genes in the pathogen and the resistance genes in the host plants need to be identified to gain further understanding of pathogen-plant interactions and to control blackleg disease effectively and efficiently in *Brassica* crop production.

There are two types of the blackleg isolates, A and B, assigned on the basis of symptoms on *B. napus* (Johnson and Lewis 1994). The A type isolates have been recently subdivided into PG2, PG3, PG4 and PGT with respects to differential reactions on cotyledons of *B. napus* cultivars including 'Westar', 'Quinta', 'Glacier' and 'Jet Neuf' (Mengistu 1991, Koch et al.1991, Kuther et al.1993 and Fernando and Chen 2003). In contrast, B type isolates have been classified into three genetically distinct subgroups: NA1, NA2 and NA3 (Kochet al., 1991).

In plants, several dozen disease resistance genes (R-genes) have been cloned and classified mainly into four groups according to the conserved domains in their protein sequences products. Resistance genes, such as Pto (*Pseudomonas syringae* pv. tomato) encoding a serine/threonine protein kinase (Martin et al., 1993), belong to the first group. The Cf (*Cladosporium fulvum*) gene family in tomato represents the second group, which encode proteins with leucine rich repeats (LRR) (Jones et al. 1994; Banerjee, 2001). The third group, such as the Xa21 (*Thasomonas oryzae*) gene in rice, codes for a structural receptor kinase with LRR motifs (Song et al., 1995). The most common R-genes in the last group have a nucleotide binding site (NBS) and an LRR domain (Hammond-Kosack and Jones, 1997).

Map-based cloning was the main strategy employed to clone the above disease resistance genes. In plants, the first R gene cloned by the map-based cloning method was the tomato PTO (Martin et al. 1993) gene. Although at least 10 genes conferring cotyledon resistance and adult plant resistance to blackleg in *B. napus* have been mapped (Ferreira al 1995, Maperhofer et al., 1997; Pilet et al., 1998; Rimmer, 1999; Rimmer et al., 1992; Zhu et al., 2003; Rimmer, 2006), none of them has been identified. For instance, Rlm1, Rlm3, Rlm4, Rlm7 and Rlm9 were mapped on linkage group 10 (LG 10) / N7 (Mayerhofer, 1997; Delourme, 2004; Mayerhofer, 2005), LepR1 on linkage group N2, LepR2 and LepR3 on N10 (Li and Cowling 2003, Yu et al. 2004, 2005), respectively. More recently, LmR1 has been finely mapped on the N7 linkage group, equal to LG10 (Mayerhofer, 2005).

In this report, the LepR3 gene in the *B. napus* cultivar, 'Surpass 400', which is a gene introgression from *B. rapa subsp. Sylvetris* (Crouch et al. 1994, Anon. 2001), was targeted through map-based gene cloning. Sequence related amplified polymorphism (SRAP) was used to construct a fine map for the LepR3 gene and a physical map was constructed with the Arabidopsis genome sequence as a reference. The candidate gene for LepR3 was identified using SNP development.

Materials and Methods

A blackleg resistant parent, “Surpass 400”, and a blackleg susceptible parent, “Westar”, were used to produce the mapping populations. In total, 908 F_2 and 2992 F_3 plants were inoculated and screened with blackleg isolates at the cotyledon stage with selfed seeds obtained from all screened plants for further analysis.

Disease resistance phenotypes were determined using blackleg isolate 87-41 spore suspension applied to cotyledons. Isolation of single pycnidiospore isolates done as per Mereno-Rico (2001). The pycnidiospores were collected from the inoculated cotyledons after the cotyledons were completely sterilized with bleach and cultured on V8 dishes. The cotyledons were punctured with fine point forceps. 10µl of the suspension was dropped on each puncture. The plants were kept at 22 C in light overnight for recovery. Then the plants were kept grown at 22 C, 16 hr photoperiod and 70% humidity in a controlled environment cabinet. When plant symptoms fully developed (in approximately 12 days), disease severity was rated using the 0-9 classification scale of Delwiche (1980). Disease severity ratings of 0 to 4 were classified as resistant while disease severity ratings of 7 to 9 were classified as susceptible. Two to three replications of all disease assessments were done, using Westar, Surpass 400 and the F1 as controls.

SRAP was performed as described by Li and Quiros (2001). A five fluorescent dye color system with ‘6-FAM’, ‘VIC’, ‘NET’, ‘PET’ and ‘LIZ’ colors were used for signal detection with ABI 3100 Genetic analyzer. ‘LIZ’ color was used for the size standard, leaving four other colors to label SRAP primers. Since SRAP is a two-primer PCR protocol, one labelled primer was combined with unlimited number of unlabeled primers.

An ultradense genetic recombination map with over 13,500 SRAP markers has been recently constructed in out lab, using 58 DH lines of Westar×ZY821. To use this resource, the mapping population of ‘Westar’ × ‘Surpass 400’ was screened using the same primers as used for the ultradense SRAP map construction. Samples from 8 resistant plants and 8 susceptible plants were used to run SRAP markers first. After a molecular marker was found to co-segregate with disease resistance trait, 32 resistant plants and 32 susceptible plants and then 64 plants from each group were tested to find the closely linked markers. Once a marker was confirmed to be linked closely to the resistance gene, it was used to find the corresponding SRAP molecular markers on the consensus map. After anchoring the closely linked molecular marker on the ultradense SRAP map, the SRAP molecular markers flanking the anchoring marker were used to find more SRAP markers for the gene.

SRAP products were loaded onto a vertical slab sequencing gel (Fisher Biotech Sequencing System). After running, the gel was stained with silver staining method (Promega, Toronto). The target markers were identified by comparing the band patterns with the ABI marker patterns. The band was cut and eluted. The DNA solution was used as template for specific PCR in order to amplify more DNA for sequencing. The standard protocol for BigDye Terminator v3.1 (ABI, California) was used for PCR product sequencing,

The marker sequences were blasted on TAIR Arabidopsis website. Some markers landed on Arabidopsis genes. Those sequences which did not match any gene in Arabidopsis were used to do primer walking until the marker sequences landed on an Arabidopsis gene.

By using the comparative information of the landed mapping markers and the Arabidopsis genes, the resistance gene in a certain region were targeted. SNPs for this region were developed and used to screen the recombinant population.

A *B. rapa* male sterile line source was used for BAC library construction. The nuclei separation was the same as the protocol of Zhang (2000). The plate, row and column pools were prepared for each 384-well plate. PCR method was used to screen the library. DNA sequencing was done using an ABI 3100 Genetic analyzer.

Results

The segregation of the LepR3 blackleg resistance gene in the ‘Westar’ × ‘Surpass 400’ progeny showed a 3:1 segregation ratio n the F_2 and a 1:2:1 segregation ratio in the F_3, indicating that one dominant resistance gene is controlling the blackleg resistance in ‘Surpass 400’.

In order to find the closely linked markers to the resistance gene, 8 susceptible F_2 plants and 8 F_2 resistant plants were initially used to run SRAP molecular markers with 384 primer pairs were used for the initial screening. Approximately 4,000 polymorphic markers were found with two of the markers, 1217Ar269 and 1217Ar167, found to co-segregate with the LepR3 resistance gene. Later another 7,000 markers were analyzed with MapMaker V3.0 to generate the initial map for the LepR3 disease resistance gene. After removing of some distant markers, 230 near markers were mapped on the new blackleg resistance map. A population of 3,900 plants was used to run some of the markers. A recombinant population (52 recombinants) was selected by comparing these markers. The marker, 0127Fr382, is the closest marker to the resistance gene on the map, showing a distance of less than 0.3 cM to the gene.

Seven markers were successfully sequenced, among which 4 markers were in this mapping region. SNP markers were developed in this region and primer walking was conducted simultaneously.

Primer walking was carried on with those primers that amplified clear bands from ‘Westar’ and ‘Surpass 400’. BAC clone A48M23 was selected and used as the first BAC clone for primer walking.

After testing the SNPs developed on the basis of the Arabidopsis genes, one SNP molecular marker was found to be completely linked to the blackleg resistance gene with its corresponding gene a kinase-LRR gene which was considered the candidate gene. Gene function for this candidate gene will be confirmed through complementary transformation.

Discussion

Map-based gene cloning allowed the identification of a candidate blackleg resistance gene in *B. napus*. SRAP, which uses

a simple PCR based protocol, was demonstrated to be an effective marker system for map-based gene cloning. By mapping preliminary molecular makers on the consensus map, other closely linked molecular markers to the blackleg disease resistance gene were found. This suggested that the consensus map used in this study is an important tool for map-based gene cloning of genes in *B. napus*.

After sequencing the candidate gene from both 'Westar' and 'Surpass 400', there were several SNPs and one insertion, suggesting that the insertion and SNPs in the candidate gene may cause the change in disease resistance. This gene is also being sequenced in other cultivars of *B. napus* and *B. rapa* to identify any allelic polymorphism. A construct with this candidate gene is under development and once developed, will be used in transformation tests. Cloning of a blackleg resistance gene in *B. napus* will speed up the cloning of other blackleg resistance genes and facilitate the cloning of other disease resistance genes. The structural and functional analyses of these cloned genes will enhance our understanding of plant disease resistance and host-parasite interactions. These resistance genes should also be very useful in Brassica breeding programs.

Conclusions

The candidate gene for LepR3 in Surpass 400 was identified and sequenced. A construct of this gene is under development. The next step is to confirm the function of this gene.

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